AD 419154

HIGH SPECIFIC ACTIVITY
LABELING OF INSULIN
WITH IODINE-131

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ABSTRACT

Two methods reported in the literature for the labeling of insulin with iodine-131 have been studied using tracer amounts of activity and the results described in this report. Although this study concerned itself with low specific activity insulin, the design of the apparatus and experimental techniques are such that high specific activity labeling may be achieved by scaling up with little modification.

The two methods studied, one described by Yalow and Berson and the other by R. W. Helmkamp and associates, differ essentially in the reagent used in the iodination, the former using iodine in basic solution and the latter iodine monochloride. Our studies seem to indicate that even when tracer amounts of radioactive iodine are used in the preparation of labeled insulin the degree of degradation is in excess of 25%. We have shown that there exists a great need for a better method of purification of labeled insulin, conventional techniques being inadequate. Of the several possibilities which suggest themselves, paper chromatography seems the most promising for further study.

INTRODUCTION

The principle objectives of this research study were to examine experimentally some of the methods reported in the literature for labeling insulin with iodine-131 and to suggest improvements or suitable alternative procedures whereby a high specific activity insulin labeled with iodine-131 showing a comparatively low degree of degradation could be prepared.

The problem was approached as a two part study consisting of (a), the method of introduction of iodine-131 into the insulin molecule and (b), the separation of the labeled insulin from degradation products and non-utilized radioiodine. The results of the study clearly point out the greater importance of the latter aspect and we feel that future research on the labeling of insulin and other proteins with iodine-131 should be directed towards the solution of this part of the problem.

There are at present a number of commercial organizations producing iodine-131 labeled proteins. Abbott[3] markets a labeled insulin with a specific activity greater than 100 μc/ug. According to Grodsky[4] and our own investigations*, analyses show that there is often from 10 to 30 percent of degraded material in these preparations. The biological activity is also low, usually about 65 percent of what one would expect of a pure sample of insulin.

Because of radiation damage, high specific activity insulin labeled with iodine-131 is particularly unstable and a certain

*See appendix

degree of protection may be obtained by adding to the preparation other proteins such as human serum albumin. There is, however, a gradual decomposition and, while initially this may be low, the preparation may become useless after a few weeks of storage. A procedure for separating labeled insulin from degraded material just before it is to be used in an experiment is essential if the results of such experiments are to be considered valid.

We have shown that ion exchange resins generally used for purification of this kind are inadequate due to the similarity of some of the degradation products to insulin and due to the delicate nature of the protein itself. We find that gel filtration, while not increasing the degree of degradation, does not adequately separate certain major degraded components. Electrophoresis and paper chromatography using veronal buffer give a partial separation but generally certain insulin-like components remain associated with the labeled protein. A greater degree of purification can be achieved, however, by means of paper chromatography using butanol: acetic acid: water[5] but the yield of pure product is low due to the great degree of degradation in the original sample. This technique seems to give the most promising results but further research is necessary to determine the best combination of solvents and other experimental conditions.

As mentioned previously, a high specific activity insulin labeled with iodine-131 may be obtained from commercial sources. For various reasons involving control and convenience it is often desirable to prepare this material in one's own laboratory. Some of the problems involved were discussed in our previous reports, primary among them being the elimination of non-utilized radioiodine. Because of the radiation damage to the protein and the increased necessity for using special remote control apparatus to protect the worker from hazard, this unreacted radioiodine is of considerable importance. When iodine in basic solution is used as the iodination reagent, this unreacted iodine accounts for more than 50 percent of the total activity. Dialysis is often used to remove it but has the disadvantages of the inconvenience of handling large volumes of radioactive solutions and the loss of significant quantities of the protein by adsorption to the dialysis bag. Because of a more efficient utilization of radioiodide when iodinemonochloride is the iodination reagent, the total unreacted iodine is less, although it still amounts to from 10 to 25 percent of the total activity.

METHODS

For the preparation of high specific activity insulin labeled with iodine-131 by the procedure described by Yalow and Berson(1) we have designed and used the apparatus shown in figure 1.

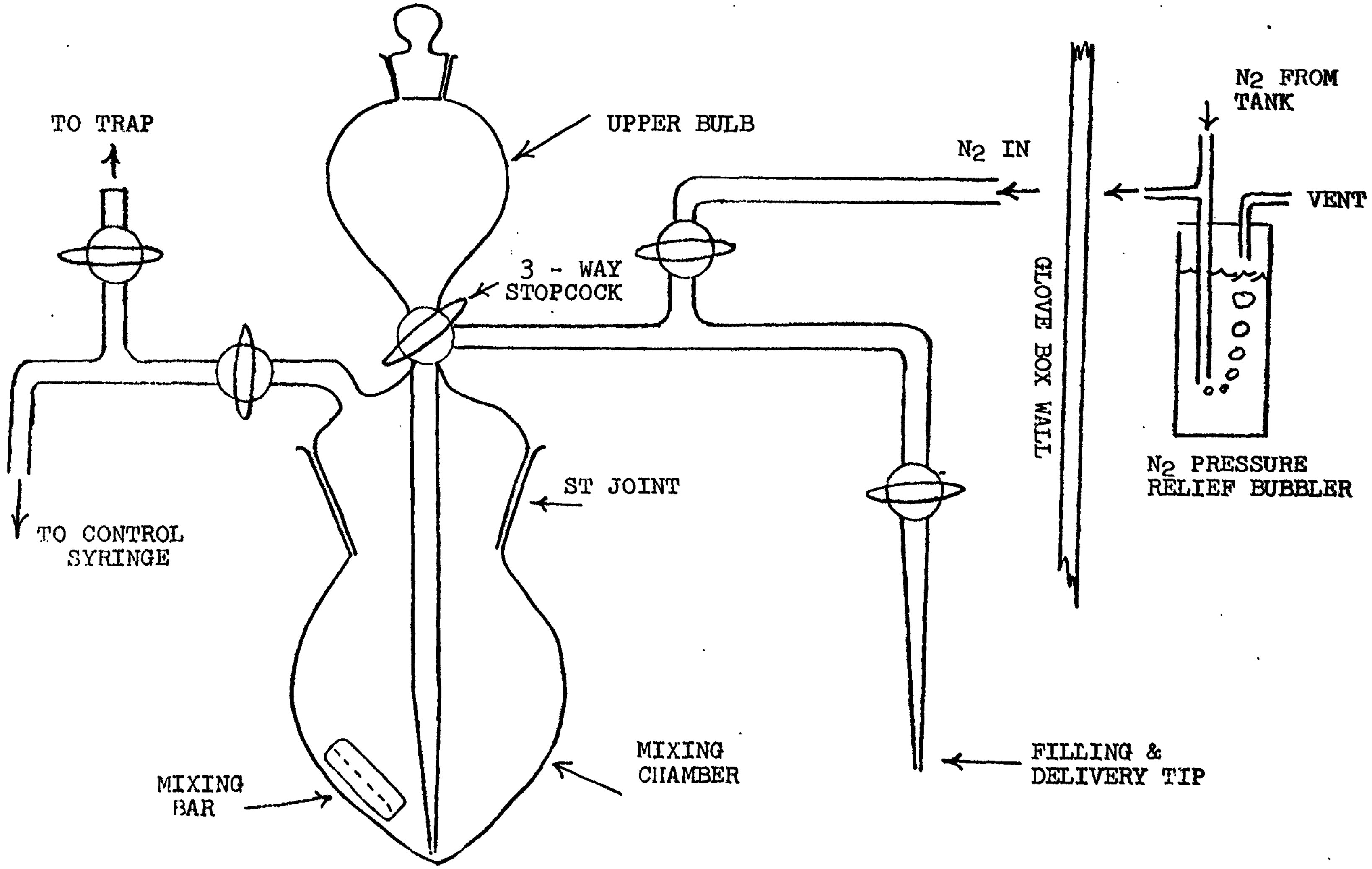

Figure 1. REACTION VESSEL FOR IODINE-131 INSULIN.

The apparatus shown in figure 1 is constructed of medium wall pyrex glass, the tubal portions of which are capillary. All stopcocks are made of teflon. The control syringe is equipped with a three way metal stopcock and attached to the apparatus through tygon tubing. The capillary tubing reaches to the bottom of the pearshaped mixing chamber. This chamber has a capacity of about 15-20 ml. and the whole apparatus is about 10 inches wide. These small dimensions allow for easy shielding with lead or lead glass bricks, one or two glass bricks being sufficient to shield the operator and allow adequate visibility. Some exposure to the hands is unavoidable, however, since the stopcocks must be turned by hand.

In the preparation, cyclohexane is introduced into the mixing chamber and the three way stopcock is opened to the filling tip. The radioactive iodine sample as received from ORNL is then drawn into the mixing chamber followed by carrier KI*. After a small wash the three way stopcock is opened to the upper bulb and sulfuric acid drawn into the chamber followed by sodium nitrite and wash. All stopcocks are closed and the reaction mixture is vigorously stirred for several minutes by means of a small bar magnet. The bottom aqueous layer is carefully removed through the delivery tip and discarded as waste and the solution of insulin in buffer brought in from the upper bulb. Reagents necessary to maintain the solution slightly basic during the reaction are also brought in through this bulb. The reaction mixture is stirred for several minutes after which the bottom aqueous layer containing the insulin is removed through the delivery tip.

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* 20 mc samples of iodine-131 as received from ORNL contains only a very small quantity of iodine and we have found that the amount of carrier must be large as compared to this or utilization will be very low. This is probably due to peroxides present in the sample which are known to inhibit the uptake of iodine by proteins. The large amount of carrier serves to increase the yield. The carrier iodine should, however, not exceed one mole per mole of insulin.

Washing during the course of the reaction is kept at a minimum to avoid excessive dilution of the sample. Care must be taken to exclude the transfer of any free iodine if gel filtration is used in the purification, since the gel bed will become iodinated. A small amount of sodium metasulphite has been used at this point to convert any free iodine to iodide [6].

The apparatus shown in figure 1 may also be used when the ICl method is employed for the preparation of iodine-131 labeled insulin. In this reaction, however, speed is of the essence and efforts should be made to carry out the entire reaction within one to two minutes. The ICl-KI reagent is unstable and after five minutes a marked decrease in iodination efficiency is observed. The more dilute ICl reagent decomposes to free iodine if kept at room temperature and must be made up fresh before each run. In this preparation the protein solution in buffer is first placed in the mixing chamber which is surrounded by ice water, no stirrer being present. The sample of KI as obtained from Oak Ridge is opened while still surrounded by the lead shield. The proper amount of ICl is added to the vial with gentle mixing. The solution is then drawn into the mixing chamber rapidly so as to cause mixing and then withdrawn for removal of inorganic iodide and further purification.

The apparatus shown in figure 2 was designed so that the preparation of insulin could be carried out under pressures less than atmospheric. Increased yields are obtained by eliminating losses due to transfers and the reduced pressure allows

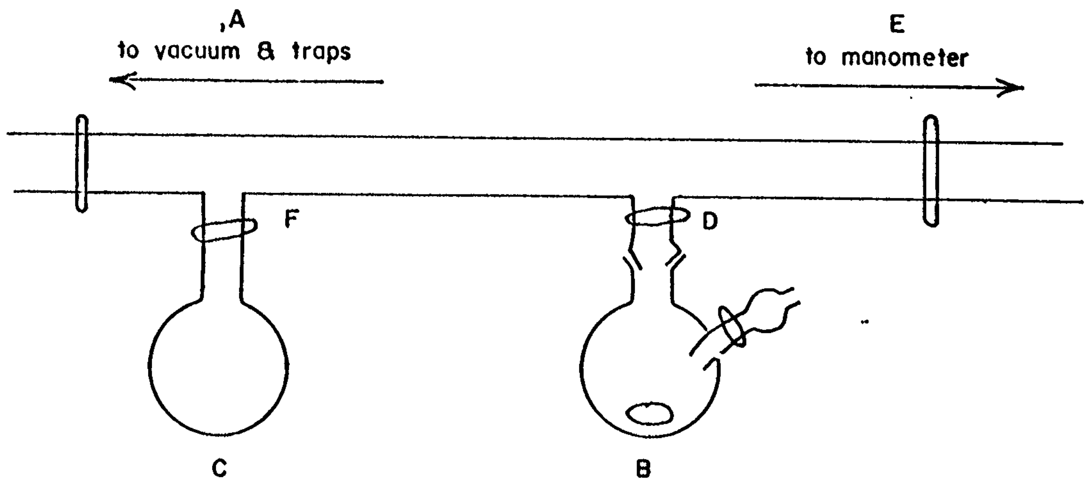

Figure 2. APPARATUS FOR REDUCED PRESSURE LABELING OF INSULIN WITH IODINE-131.

for better control of the radioactive iodine. The method of preparation is as follows: a) The system is evacuated to less than 1 mm pressure and stopcocks A and E closed, isolating the reaction vessel and storage flask B and C. The solvent and reagents are then introduced into vessel B through the dropping funnel, B being closed off at stopcock D. The mixture is stirred, the iodine passing into the cyclohexane layer. While the reagents are introduced the reaction flask is kept cold so that upon entering they are frozen. b) After a few minutes stirring, the stopcocks at D and F are opened and the liberated iodine, cyclohexane, and some water are distilled into storage vessel C which is immersed in liquid nitrogen or dry ice-acetone mixture. This serves as a purification step for the iodine. Upon completion of the distillation, which takes only a few minutes, the stopcock at F is closed and the reaction vessel B removed from the line. Assay of this residue less that of the original activity gives the value of the iodine in C. c) The reaction vessel B is cleaned and reattached to the line at D. (A duplicate vessel may be used at this point.) The system is again evacuated to less than 1 mm. While stopcock F and D are open the iodine, cyclohexane, and some water are distilled from C to B by removing the cold bath from C and placing it under B. Upon completion of the distillation D is closed and B brought to room temperature or below. (10 - 20° C). At this time the protein solution is run from the dropping funnel and the mixture agitated by means of the bar magnet. d) After a few minutes of agitation

D and F are opened, and the excess iodine, cyclohexane and some water are distilled into vessel C for reuse or storage. B is removed from the line and the protein solution is ready for further purification.

The procedure used in iodinations with ICl is essentially that described by Helmkamp and co-workers.[2] In our tracer runs the radioiodide was used just as it was received from Oak Ridge National Laboratories.

Iodine Monochloride Reagent

The iodine monochloride reagent was prepared as the above authors describe, namely, by adding 21 ml of concentrated hydrochloric acid to a solution of 0.555 gm of KI, 0.365 g KIO_3 and 29.23 gm NaCl and bringing the final volume to 250 ml with distilled water. The excess iodine was removed by extracting the solution 10 times with about 10 ml portions of carbon tetrachloride until the latter was colorless. Air passed through a trap containing water was used to remove last traces of $C\ Cl_4$. The exact molarity of the solution was determined by adding an excess of KI and titrating the liberated iodine with standardized thiosulphate solution. Such a solution is approximately 0.02 M in ICl. The more dilute solution, made up just before reaction and kept ice cold, was prepared by diluting 1 cc of the above to 50 ml with 2 M NaCl.

Insulin

The insulin solution was prepared from a sample of crystalline beef insulin obtained from California Corporation for Biochemical Research and was prepared by suspending the material in distilled water and adding 1 drop of 1 N HCl. The concentration used in our experiment was 1 mg/ml.

Borate Buffer

Borate buffer was prepared by adjusting a distilled water solution of 0.32 M NaCl and 0.4 M H_3BO_3 to pH 7.5 (pH meter) with NaOH to a final concentration of 0.08 M in NaOH.

Preparation

In the preparation 0.1 cc of the insulin solution was added to 0.5 cc of borate buffer of pH 7.5, 0.1 ml of the iodine monochloride reagent was added to the Oak Ridge sample of radioiodide (0.5 cc) after having added 0.5 cc borate buffer of pH 7.5. This was immediately added to the insulin solution and mixed.

Solvents for Chromatography

The veronal buffer of pH 8.6 was prepared by diluting to one liter 13.68 g barbital and 20.60 g sodium barbital. The butanol: acetic acid: water was 1-butanol, 12 ml; water, 5 ml; acetic acid, 2 ml.

Discussion of Results

A number of preparations of labeled insulin were made using ICl as the iodinating reagent and there is much in favor of this procedure. This method gives the best results as to yield of labeled insulin and efficient utilization of iodine-131. In our studies trace amounts of iodine-131 were used but the scaling up can be achieved with little change in experimental conditions. It appears that one of the influencing factors in yield of labeled protein is the intimate contact of the reactants during iodination. This appears to contribute to the high yield in the preparation of labeled growth hormone described by Hunter and Greenwood [6]. These workers were able to obtain very high specific activity material of high purity and yield by using an organic reagent, chloroamine-T, to convert iodide to iodine. This method may also be used to prepare labeled insulin but there is not much advantage over the iodine monochloride method since the problem of separating the degradation products is still with us.

Figure 3 shows the scan of a chromatogram made immediately after preparation by the ICl method using trace amounts of activity and following the procedure described above. This scan shows only two peaks, A and B. The peak near the front is inorganic iodide and that at the origin is labeled insulin along with other labeled materials which appear to be similar to insulin. The chromatogram was run in veronal buffer at pH 8.6 in the apparatus shown in Fig. 4. When all the conditions of the preparation were observed, this pattern could always be reproduced. Figure 5 shows a scan of the same material run in butanol: acetic acid: water. Here we see at least 4 peaks, the largest of which is labeled insulin and accounts for almost

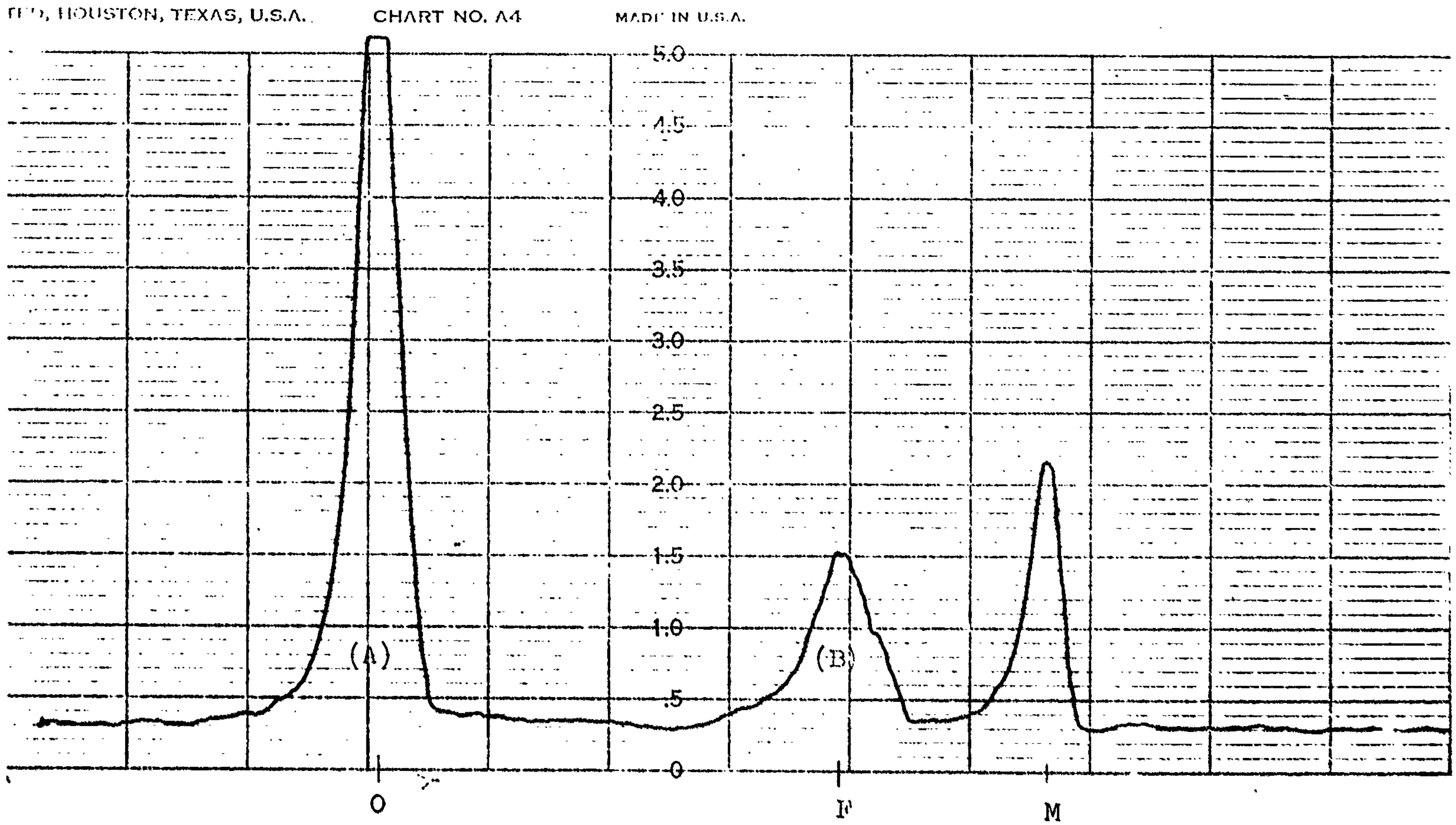

Fig. 3 I-131 Insulin Chromatogram (Veronal)

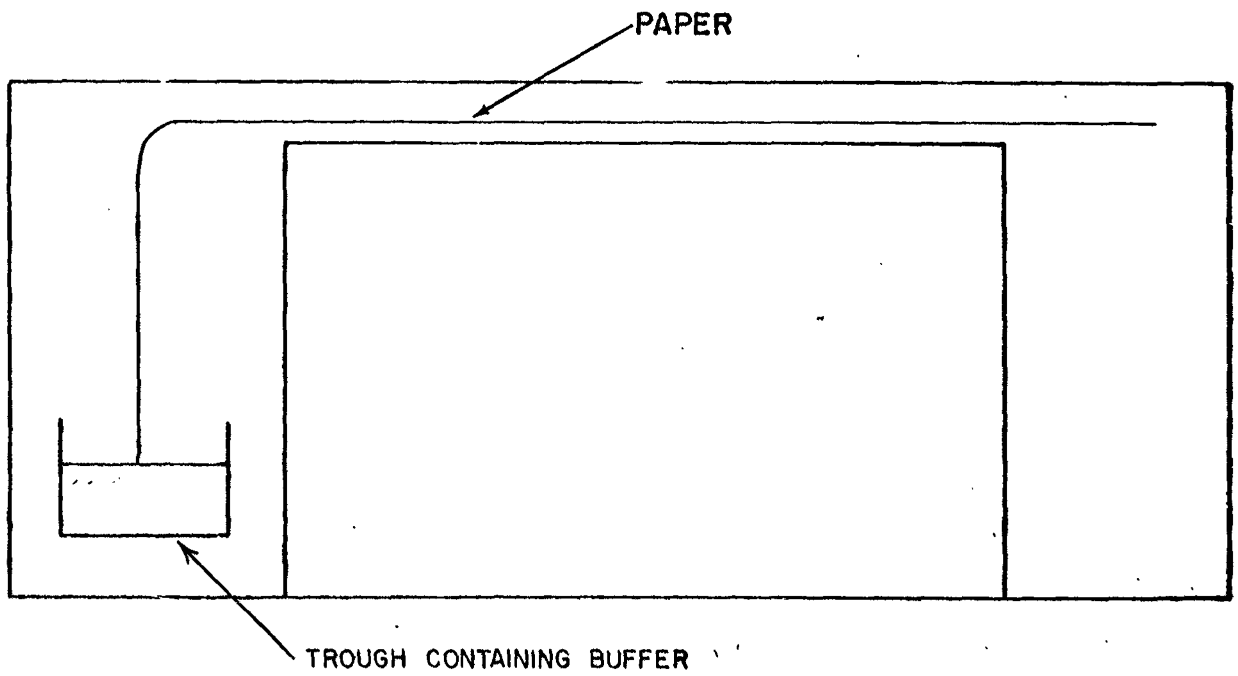

FIGURE 4. CHROMATOGRAPHY APPARATUS

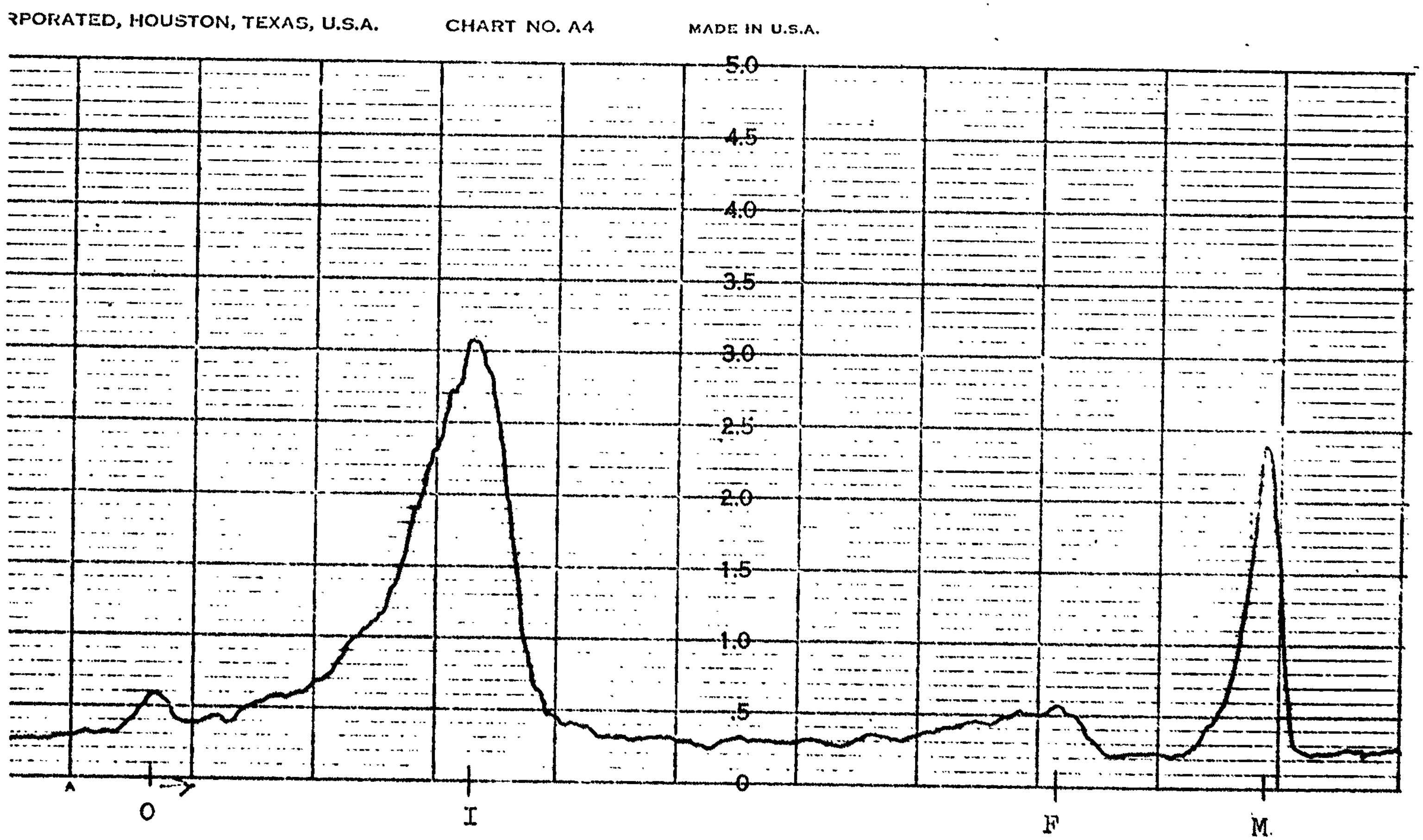

Fig. 5   I-131 Insulin Chromatogram (Butanol-Acetic)

50 percent of the total activity. The chromatogram run in veronal buffer, the scan of which is shown in Fig. 3 was rerun in butanol: acetic acid: water. This scan is shown in Fig. 6 and has the same general pattern as the original in this system. The separation in the latter solvent system, although not complete, suggests that a pure sample of insulin can be obtained from such a preparation.

Many attempts were made to separate the several labeled components shown to exist in the above preparations using column chromatography. Varying results were obtained during these studies. Sephadex G-25 and G-50 do not achieve a separation of all of these materials as can be seen from the two scans in Fig. 7 and 8. The scan in Fig. 7 shows a preparation from which the inorganic iodide has been removed, before passing it through the Sephadex column. Fig. 8 was made from a chromatogram of the same material after passage through the column. The pattern is essentially the same and a count of the column showed that very little activity remained adsorbed to it.

In Fig. 9 and 10 we see two scans of chromatograms, the first of which was run immediately after preparation, and the second of the same material after passing through a desalting resin AG 11A[7]. It can be seen that this resin actually decomposes the insulin further and therefore is not recommended for removal of salts. The activity of the degradation products exceeds even that of the insulin.

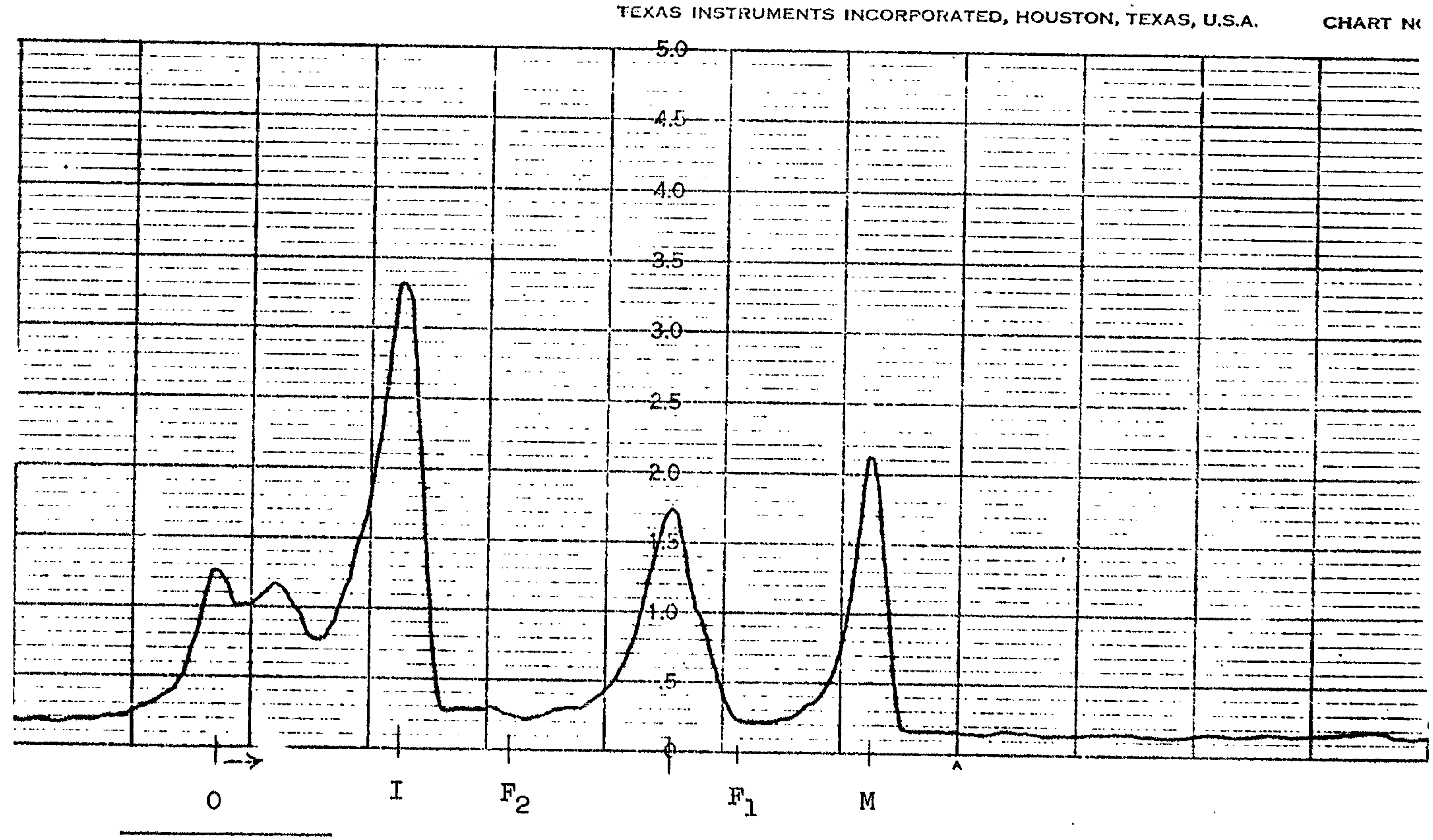

Fig. 6 Veronal Re-Run in Butanol Acetic

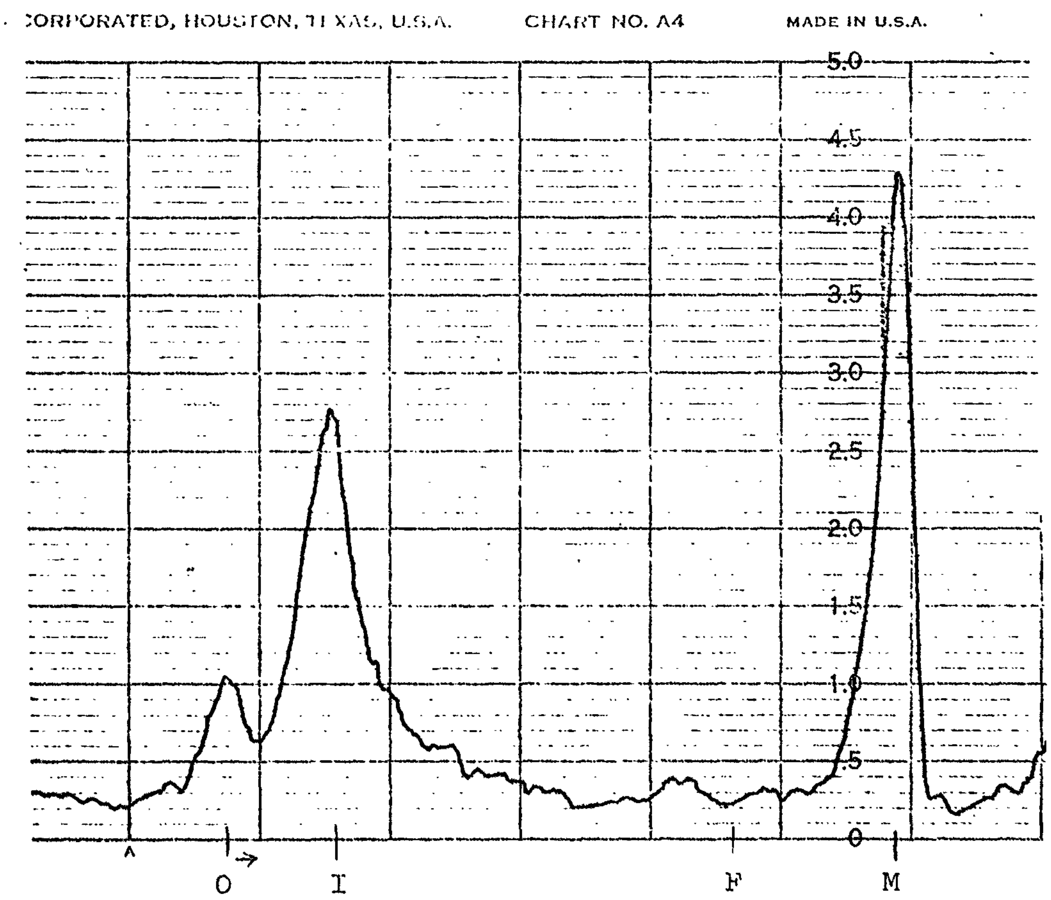

Fig. 7 I-131 Insulin Before Column Chromatography

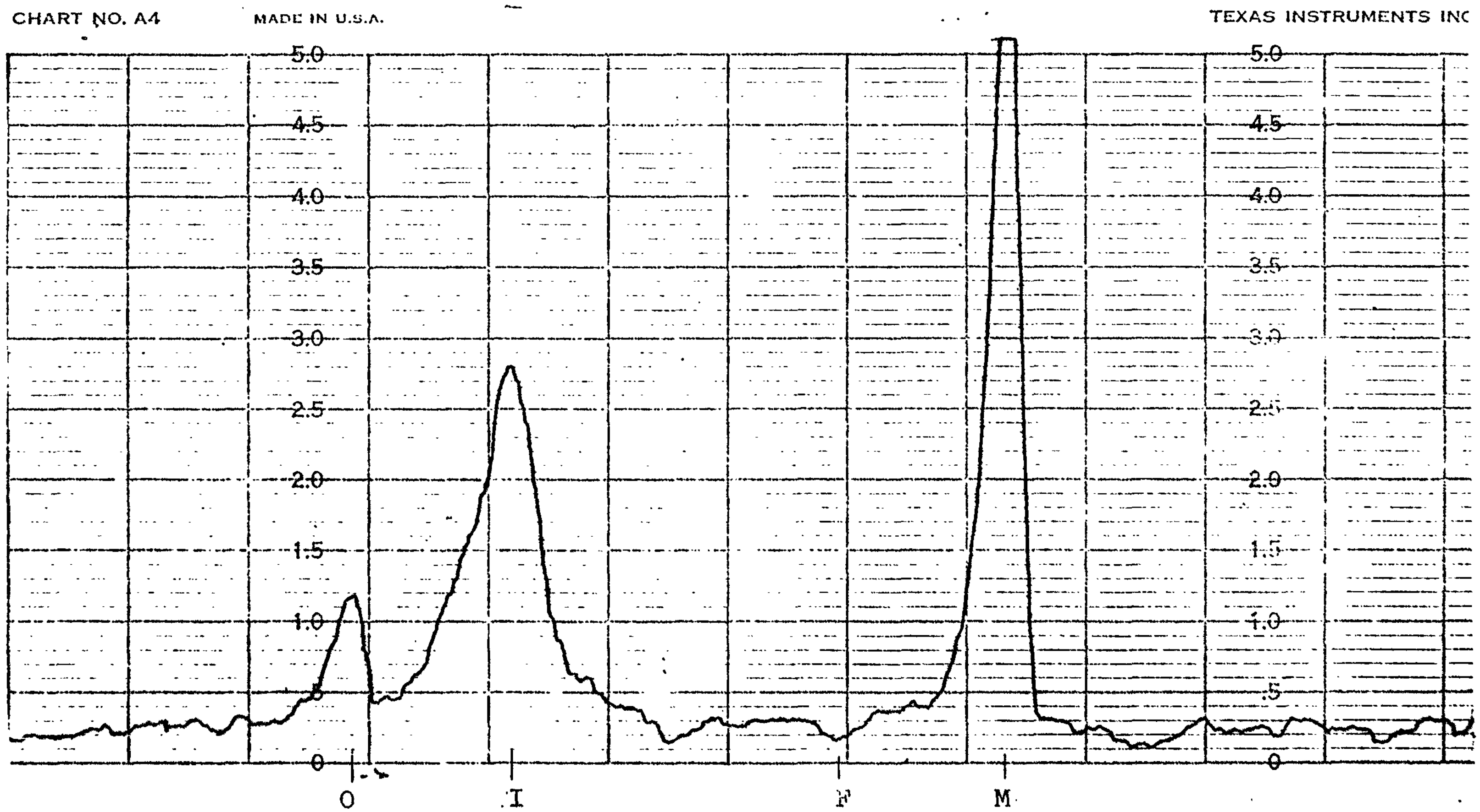

Fig. 8 I-131 Insulin After Column Chromatography

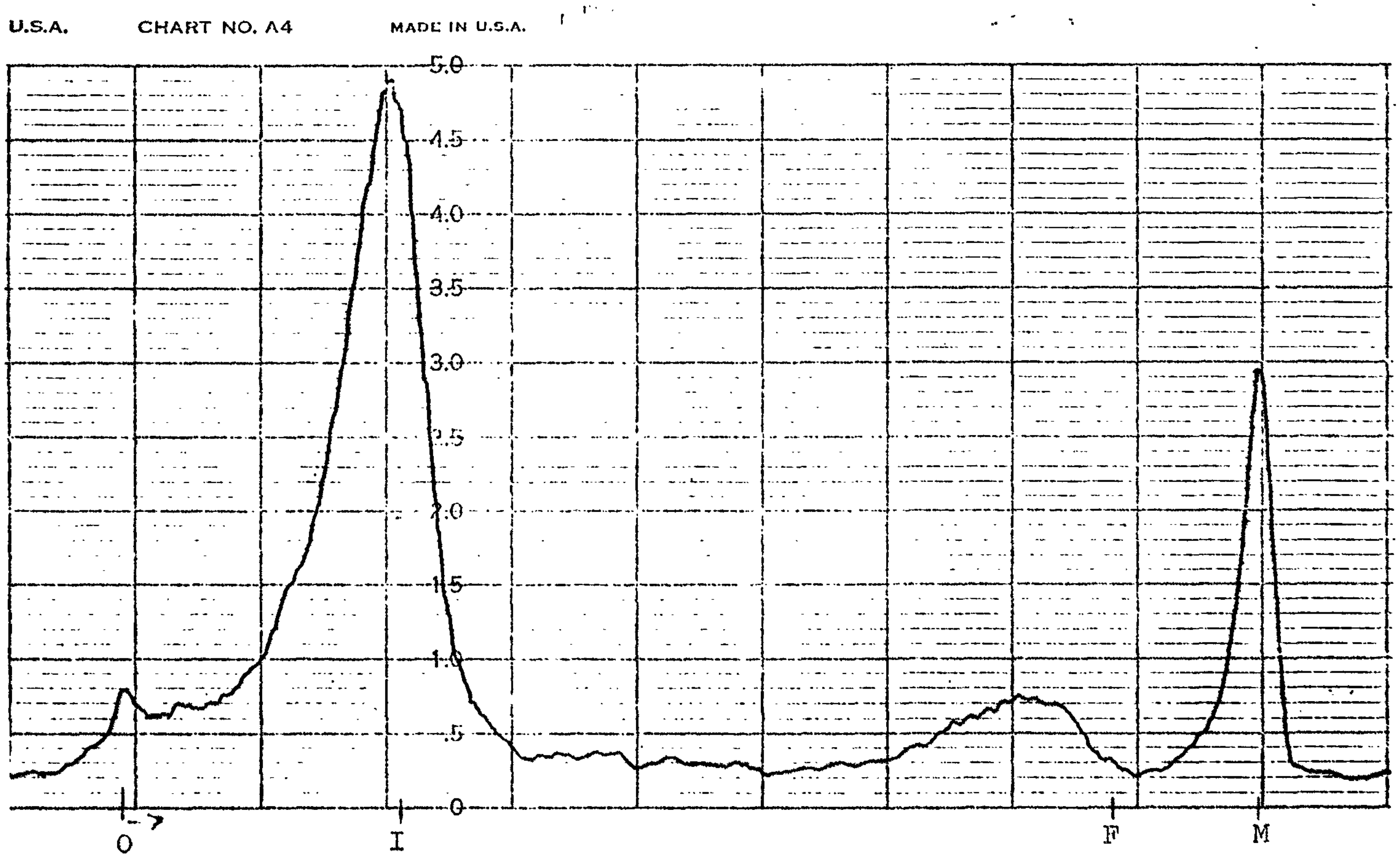

Fig. 9 I-131 Insulin Before Desalting

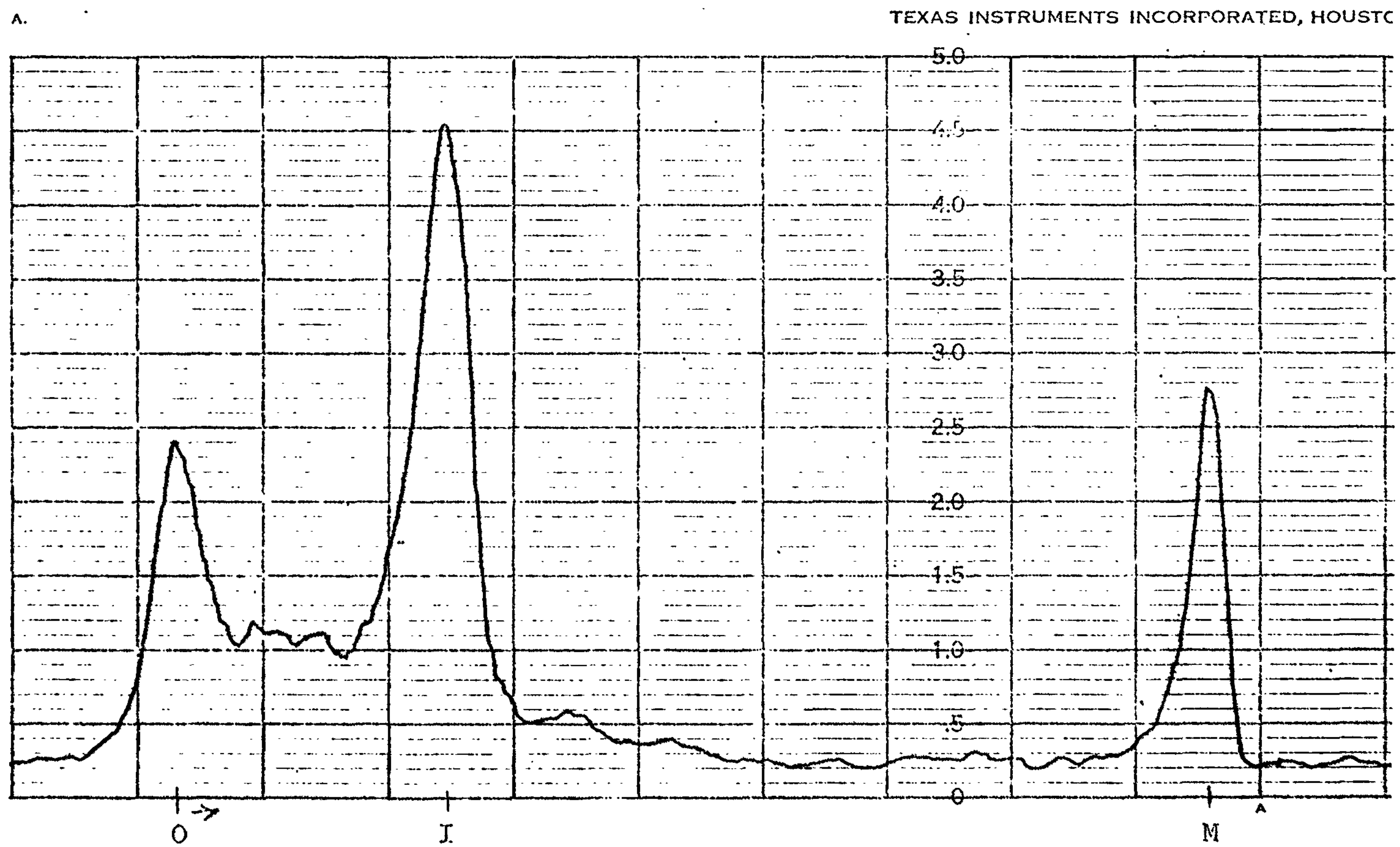

Fig. 10 I-131 Insulin After Resin Desalting

The data in Table 1 shows an attempt to separate a sample of labeled insulin using a Sephadex G-25 column 30 cm long and 1 cm in diameter. Even though there is evidence of a separation the 25% of material always remaining associated with insulin is found in the first peak, the second being mainly inorganic iodide. This result is shown in the scans in Fig. 11 and 12.

When the insulin preparation is chromatographed with the addition of human serum albumin to the Sephadex column prior to use the results are the same whether in veronal buffer or butanol: acetic acid: water. There is very little difference if the starting insulin is freshly prepared or allowed to stand refrigerated for several weeks.

On the basis of the above information, if the iodine monochloride method is used one can expect to lose 25 percent of the activity in degraded products on the trace level, and probably more when the procedure is used in the preparation of a high specific activity material. There are apparently two types of degradation products: those of low molecular weight when compared to insulin, and those having molecular weights similar to insulin. The former may be separated from the preparation by paper chromatography with veronal buffer and generally move ahead of insulin; the latter are only incompletely separated on paper using butanol: acetic acid: water.

Essentially the same results are obtained when the iodination is conducted using free iodine as the iodinating reagent[5].

TABLE I. Separation of I^{131}- Insulin with Sephadex G-25

Fraction No.	Activity (CPM)	Notes
1	326	(1 ml fractions were counted with NaI crystal)
2	310	
3	329	
4	5,185	
5	301,369	
6	over 1,000,000	(Insulin and Degradation Products. Fig. 11 is fraction 8)
7	" "	
8	" "	
9	" "	
10	621,745	
11	370,180	
12	202,916	
13	324,382	
14	over 1,000,000	(Mostly inorganic with some insulin. Fig. 12 is fraction 16)
15	" "	
16	" "	
17	" "	
18	203,720	
19	80,587	
20	48,579	

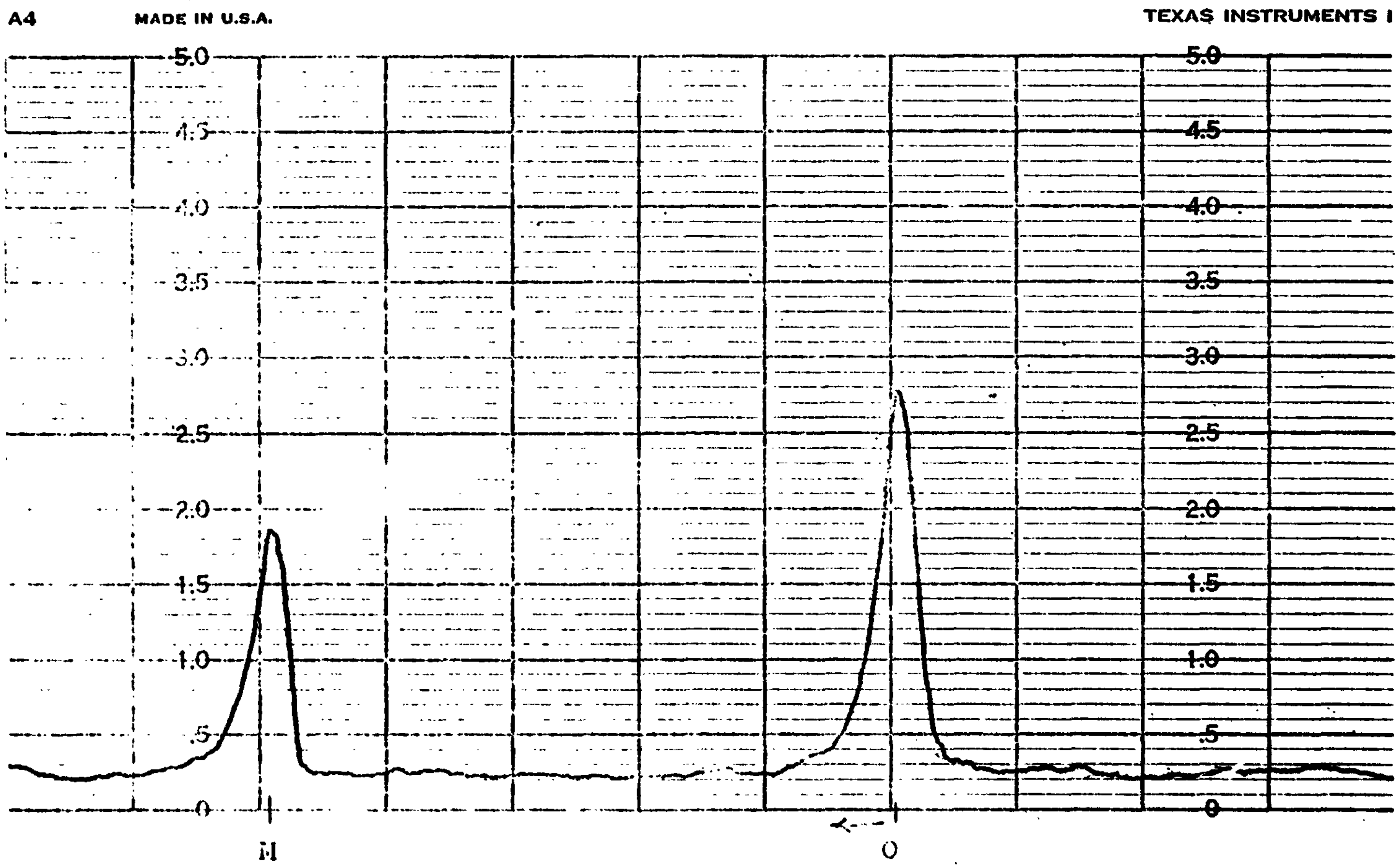

Fig. 11 Fraction Showing Single Insulin Peak

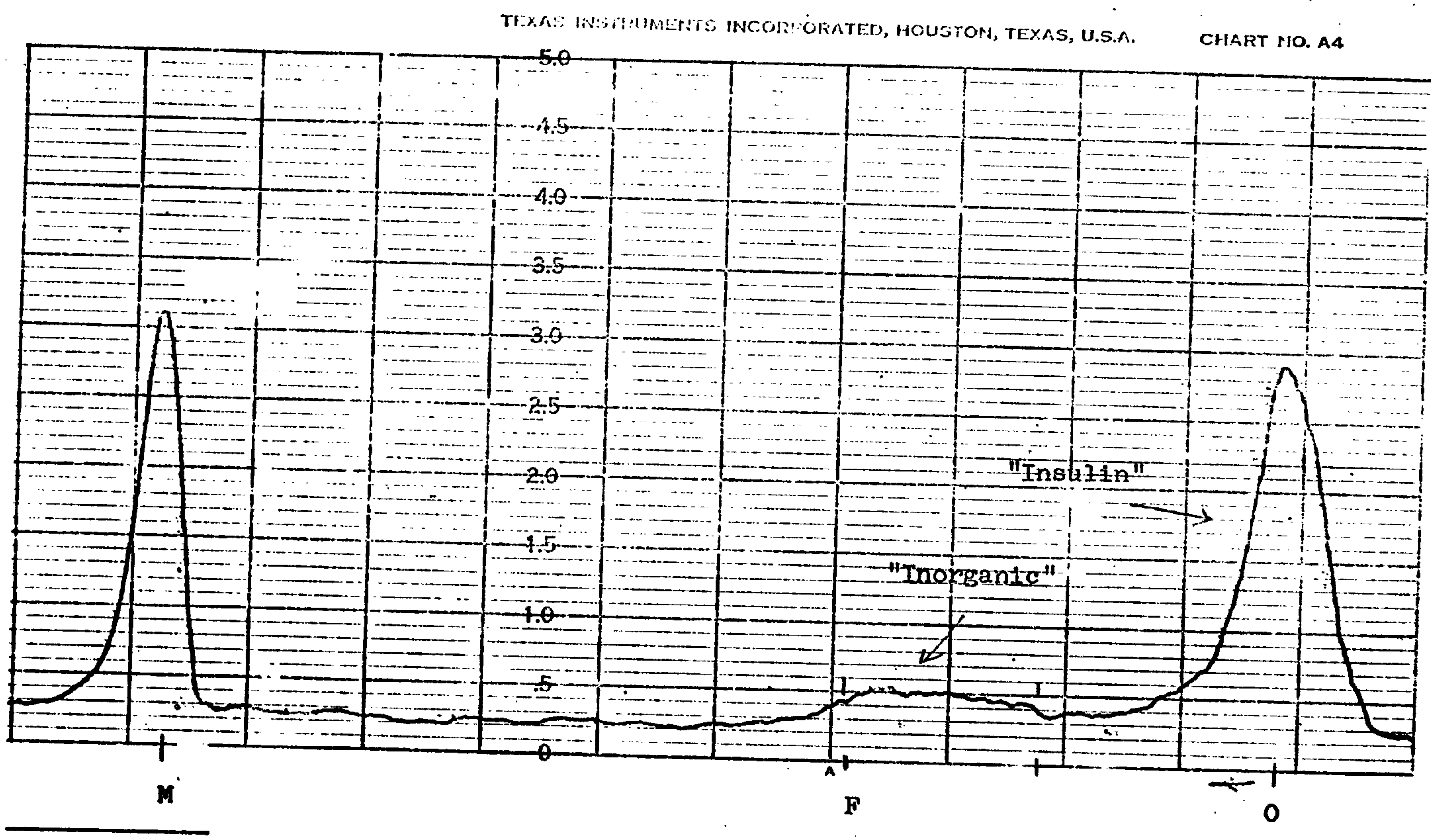

Fig. 12 Fraction Showing Incomplete Separation

This seems to indicate that a large percentage of the insulin preparations being used in various studies is a mixture of labeled insulin and products having a similar molecular weight as insulin. More than likely this accounts for the loss in biological activity of the insulin noted in the literature [5].

The behavior of proteins in general and insulin in particular on cellulose suggest that this medium offers possibilities of obtaining a pure sample of insulin labeled with iodine-131. It has been suggested that insulin has a great affinity for this material and that considerable losses are observed when such columns are used in its purification[1]. We feel that the losses in radioactive material are probably due to the greater affinity of the degraded products rather than insulin itself. In other words, with the proper selection of solvents insulin may be eluted from a cellulose column and, under ideal conditions, may be obtained in pure form. To demonstrate this point the following experiment was carried out using Sephadex: Insulin labeled with iodine-131 was prepared as described above by the iodine monochloride method. This preparation was put on a column of Sephadex G-25 of about 1 cm in diameter and 24 cm in length and washed through with borate buffer of pH 7.5. Only traces of activity were removed from the column even after the addition of 50 ml of this buffer. A scan of the column indicated that the bulk of the activity remained near the top. Another preparation of labeled insulin was made in the same manner, placed on a similar column as before, and elution was started with physiological saline containing 1 drop of normal hydrochloric acid per 250 cc of solution. Again, only traces of activity were removed from the column after 50 ml of wash. A third preparation was made, placed on a similar column, and eluted with physiological saline. Here two major peaks were obtained (shown in TableII) with the insulin and some degradation material in the first peak and the inorganic in the second. Fig. 13 shows the scan of a chromatogram of this material in veronal buffer immediately after preparation, Fig. 14 shows a scan from vials 3, 4 and 5(A) and Fig. 15 shows that of vials 6,7,8[13].

In addition to the above observations we also conducted the following experiment from which certain interesting conclusions may be drawn. A number of chromatograms were run on Watman # 1 paper in veronal buffer pH 8.6 and butanol: acetic acid: water. As pointed out before when veronal buffer is used as solvent certain degradation products remain at the origin with the insulin (usually about 25 percent of the activity). When the same chromatogram is run in butanol: acetic acid: water there is a partial separation of the insulin from the other materials. These experiments indicate that materials which remain bound at the origin are degradation products rather than insulin. We may conclude, then, that cellulose may be used effectively for the purification of a labeled insulin preparation. The following experiment is suggested for future experimentation: A column of Watman # 1 paper in powder form will be pre-treated with veronal buffer at pH 8.6. On this column will be added a preparation of insulin labeled with iodine-131 as described above and eluted with veronal

TABLE II. Separation of I^{131}- Insulin on Sephadex G-25

Fraction No.	Activity (CPM)	Notes
1	389	(1 ml fractions were counted with NaI crystal)
2	41,077	
3	over 1,000,000	(Mostly Insulin and Degradation Products Fig. 14 is fractions 3,4,5)
4	" "	
5	" "	
6	" "	(Mostly inorganic. Fig. 15 is fractions 6,7,8)
7	391,586	
8	173,464	
9	34,274	
10	14,677	

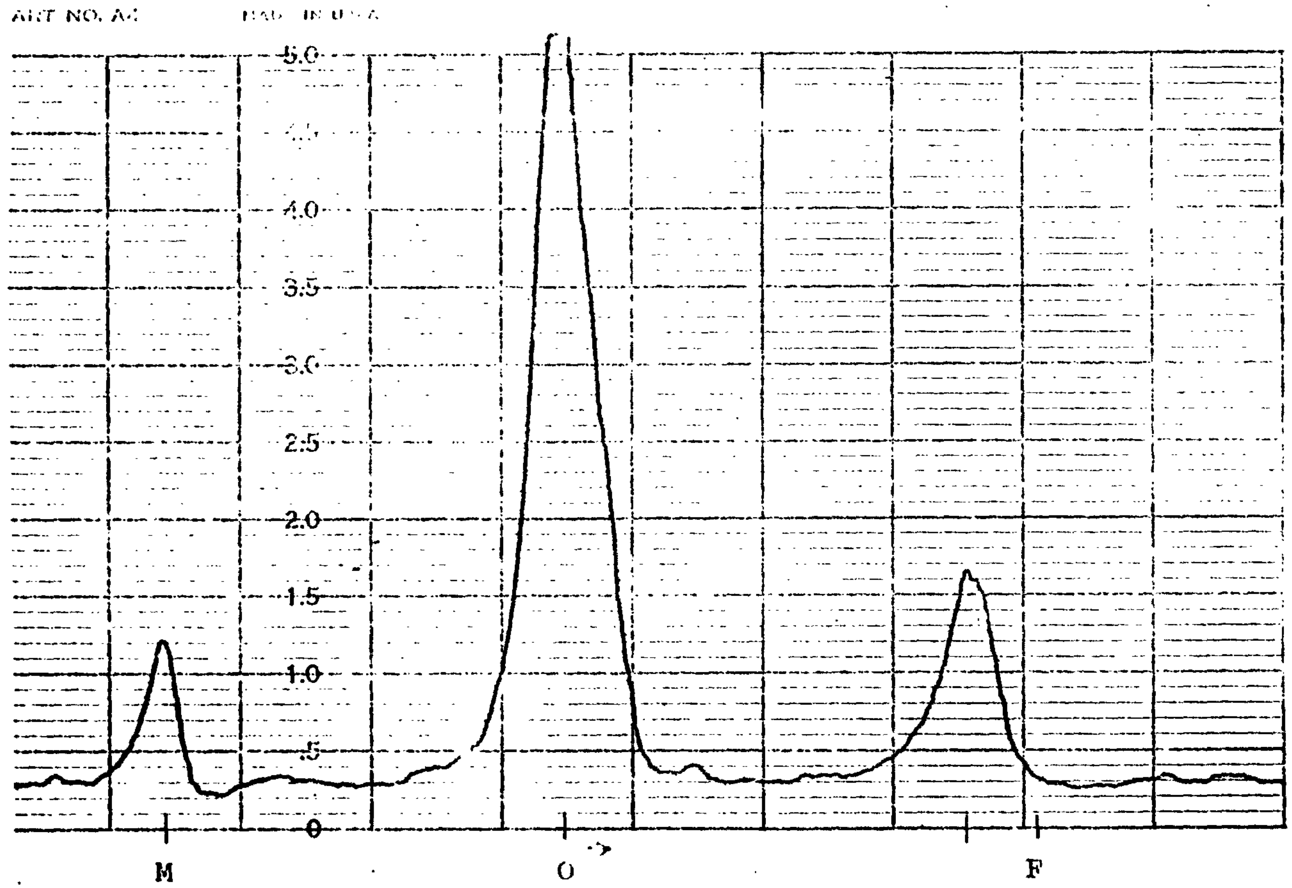

Fig. 13 I-131 Insulin Before Sephadex G-25 Purification

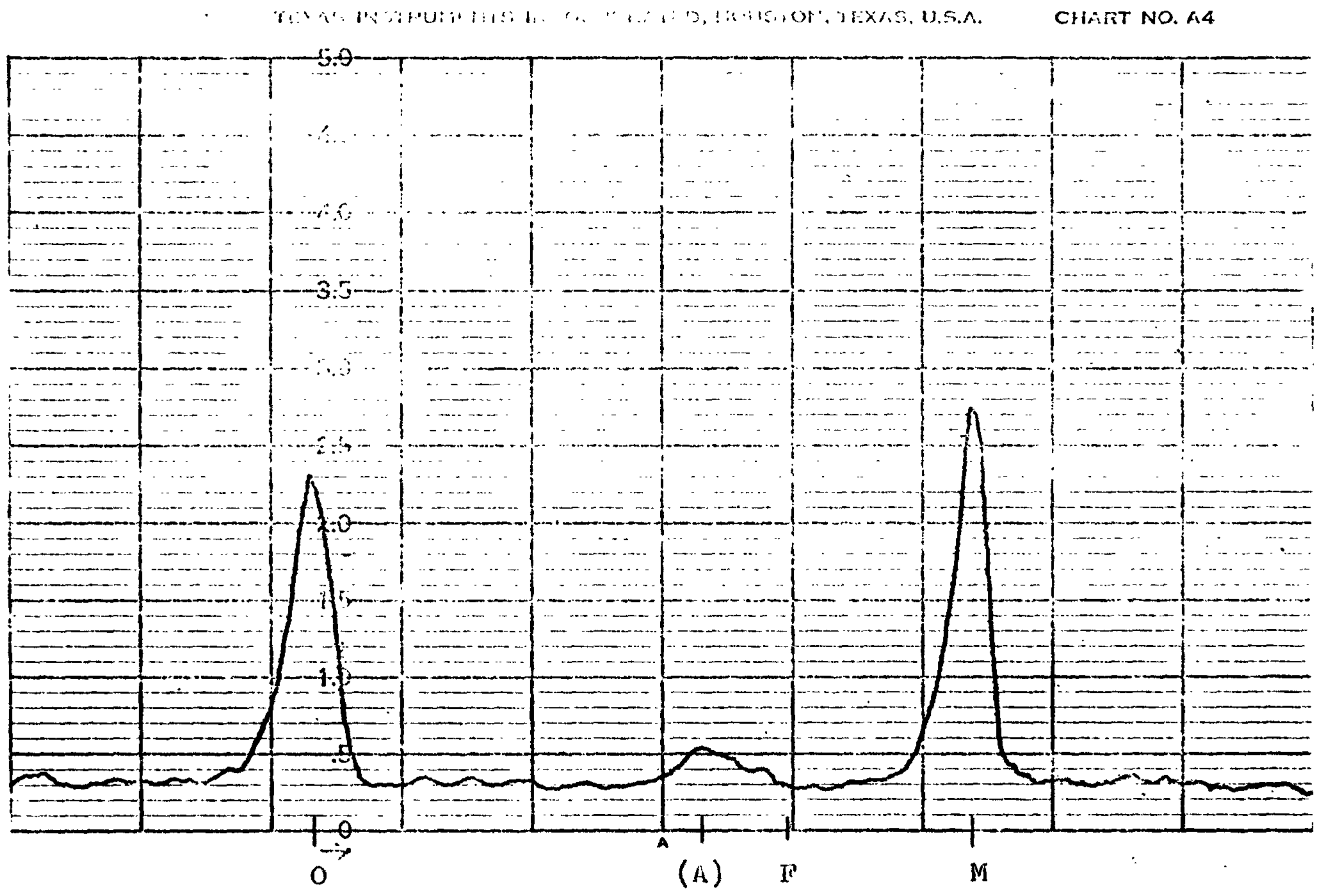

Fig. 14 Fraction Showing Non-Separation of Impurities (A)

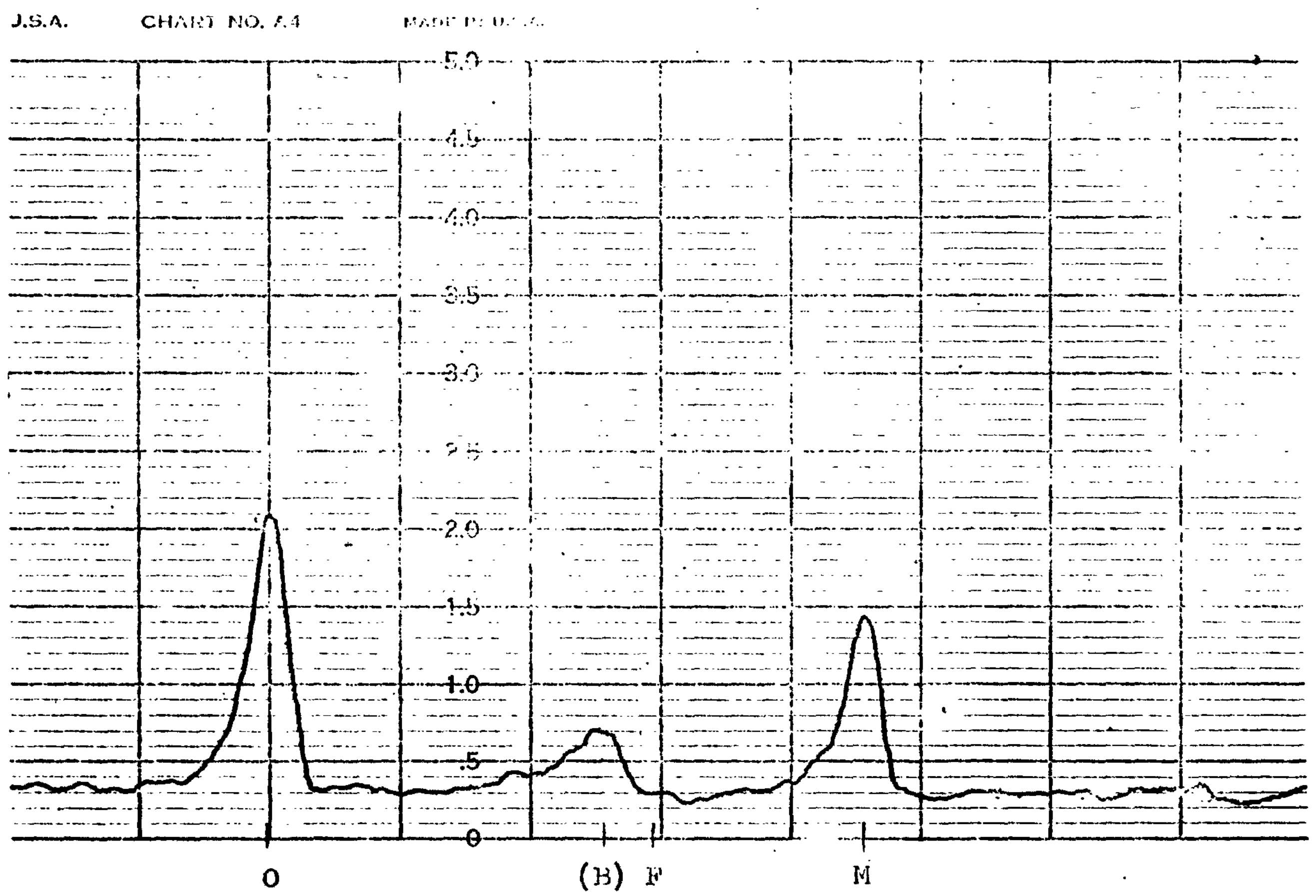

Fig. 15 Fraction Showing Non-Separation of Impurities (B)

buffer. The labeled insulin with certain degradation products should remain on the column and the inorganics and certain other degradation products should be eluted. An elution will then be carried out using butanol: acetic acid: water. Temperature control may be an important factor in this elution and certainly after the data obtained so far, a certain measure of purification of the insulin may be expected.

SUMMARY & CONCLUSIONS

A study of the two most frequently used methods for the labeling of insulin with iodine-131 is presented. The iodine monochloride method is shown to give better results than that which employs iodine in basic solution. More than 25 percent of the initial activity used in a typical preparation with iodine monochloride appears as degradation products remaining at the origin with the active insulin when chromatograms are run in butanol: acetic acid: water. The study suggests that improved techniques are needed for the separation of labeled insulin from degraded components. The inability to achieve this is probably responsible for the conflicting reports in the literature as regards the results obtained with preparations of I-131 insulin.

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(7) Bio-Rad Laboratories, Richmond, California

APPENDIX

As mentioned in the body of the report the radio-iodoinsulin of high specific activity obtained from Abbott shows a high degree of degradation. Our data seem to indicate that some of this is probably due to the method used in separating the inorganic iodide. Ion exchange resins tend to increase the proportion of labeled components having similar properties as insulin at the expense of the insulin itself.

Fig. 16 shows the scan of a chromatogram of Abbott radio-iodoinsulin using veronal buffer pH 8.6 as solvent. There are two major peaks, the largest of which is at the origin. The peak near the front is presumed to be inorganic iodide since our experiments show that the latter moves with the front. Fig. 17 shows the scan of a chromatogram of the same material run in butanol: acetic acid: water and shows at least four peaks. The peak marked I corresponds to insulin. This means that more than 65 percent of the activity is associated with materials other than insulin. In no preparation which we have made to date have we observed such a high degree of degradation.

Fig. 18 shows a typical glovebox set up for high level iodinations and Fig. 19 a typical column for chromatography at or near 0°C.

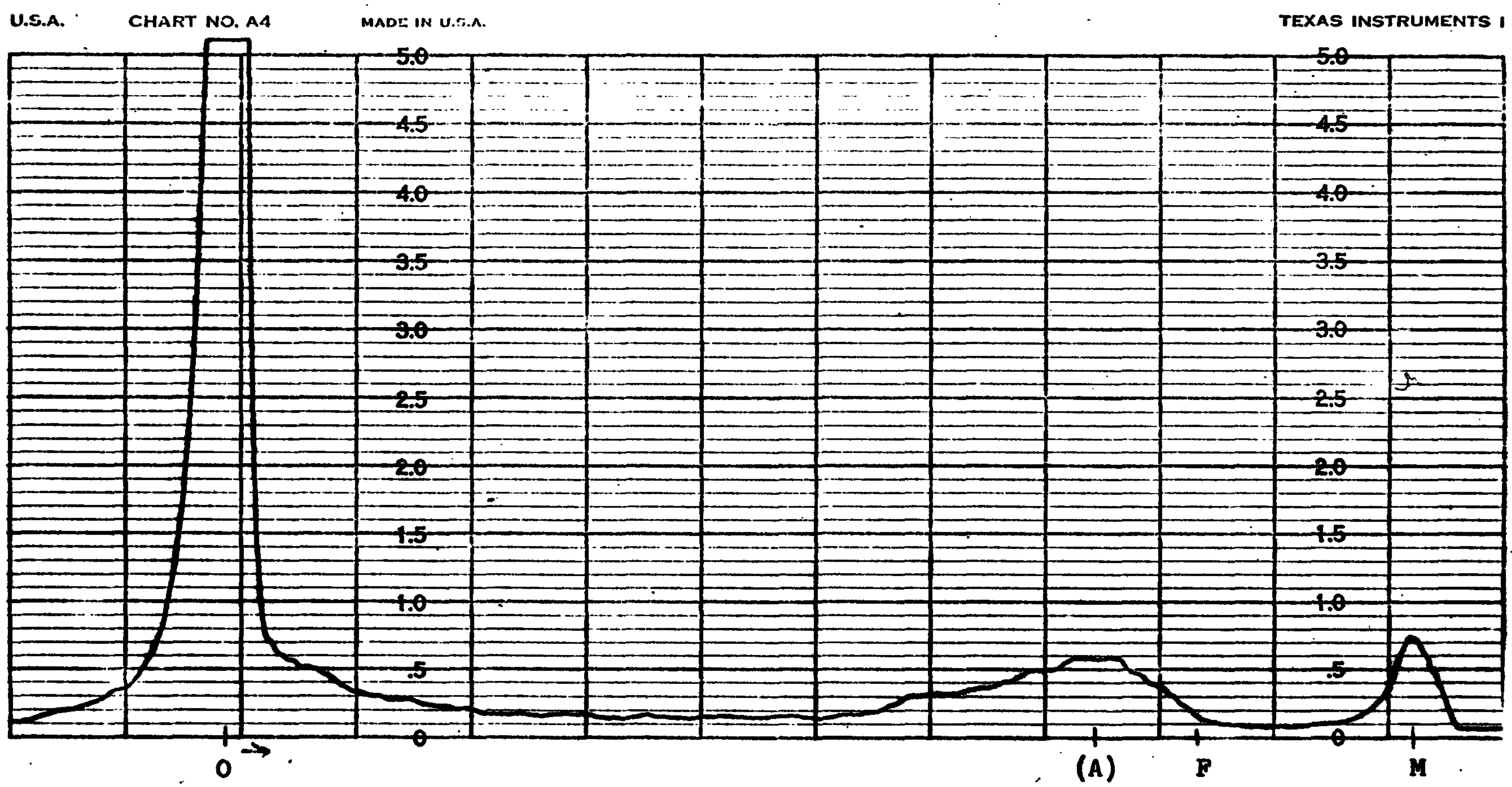

Fig. 16 Abbott # 7791 Insulin in Veronal Buffer

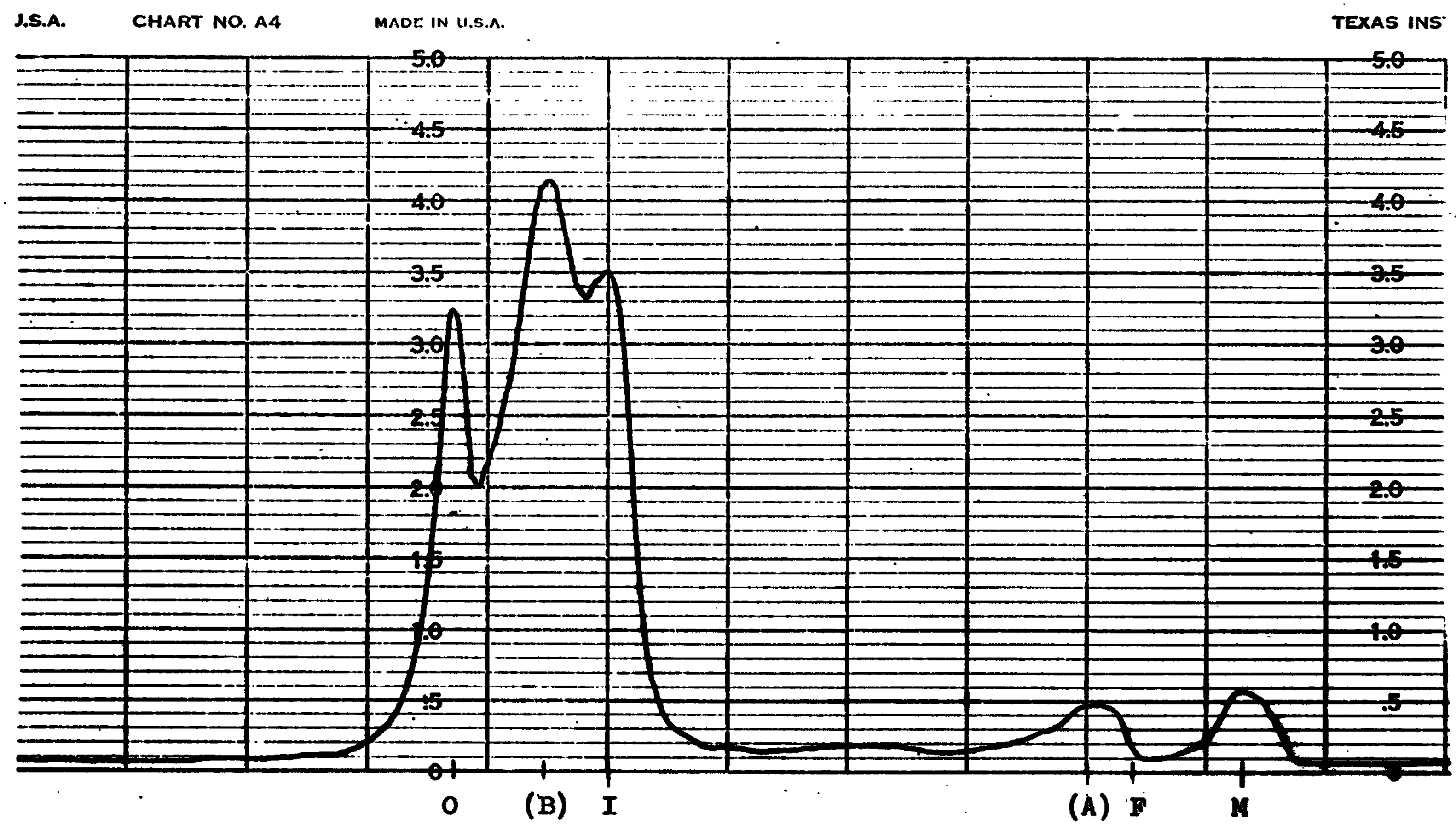

Fig. 17 Abbott Material Showing Degraded Components (A), (B) (Butanol Acetic)

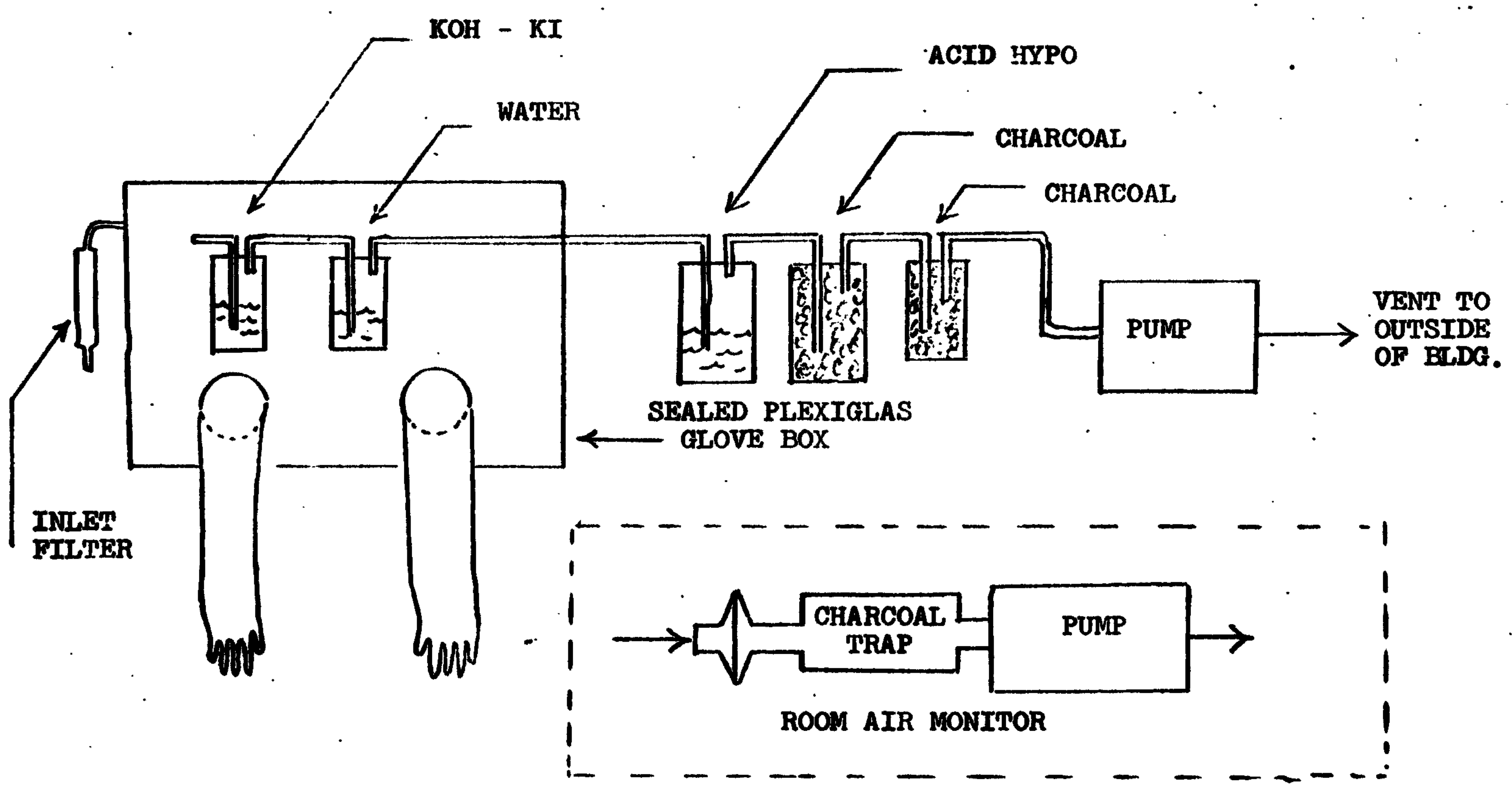

Figure 18. AIR FILTER TRAIN FOR I-131 INSULIN

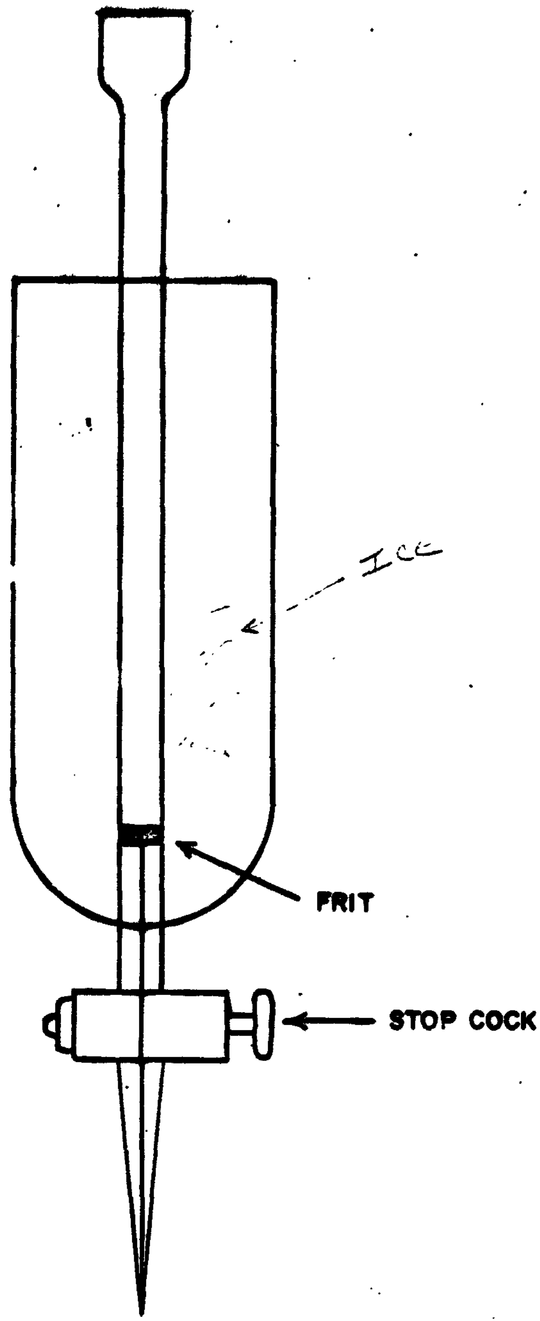

Fig. 19 Low-Temperature Chromatography Column

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